

Amendments to the Specification

Please replace the paragraph bridging pages 10 and 11 with the following paragraph:

E3 ubiquitin ligases are members of the ubiquitination cascade that transfer ubiquitin to specific substrates, rendering the substrates targets for proteasome-mediated degradation. Known HECT E3 ubiquitin ligases include, for example, WWP1 (Pirozzi et al., J. Biol. Chem 272:14611-16, 1997), E6-associated protein (E6-AP; Huibregtse et al., Mol. Cell. Biol. 13:775-84, 1993), Rsp5 (Huibregtse et al., Proc. Natl. Acad. Sci. USA 92:2563-67, 1995) and Nedd4 (Staub et al., EMBO J. 15:2371-80, 1996). Other HECT E3 ubiquitin ligases may be identified based on sequence similarity to known proteins and/or the presence of functional properties of HECT E3 ligases. A variety of techniques may be used to evaluate sequence similarity. One such technique is searches of sequence databases (e.g., ~~GenBank~~ GENBANK™). Such searches may be performed using well known programs (e.g., NCBI BLAST searches), and proteins that display high levels of sequence identity and/or similarity are candidate HECT E3 ligases. Alternatively, techniques employing low stringency hybridization may facilitate the identification of a HECT E3 ligase. Within such techniques, a known HECT E3 ubiquitin ligase (or a portion thereof) is used as a probe to screen a library (cDNA or genomic) for hybridizing sequences. Suitable low stringency hybridization conditions include, but are not limited to, 1.0 X SSPE or SSC, 0.1% SDS, 50°C. Yet another technique for evaluating sequence similarity employs PCR reactions that are performed using degenerate primers that encode a conserved sequence.

Please replace the paragraph bridging pages 16 and 17 with the following paragraph:

By way of example, one polypeptide (i.e., a WW domain polypeptide or PY motif polypeptide) may be immobilized through non-specific interactions (e.g., to a polystyrene plate) or through a protein tag interaction (e.g., an interaction between a His₆-fusion protein and a nickel plate). The polypeptide may be immobilized by, for example, contacting a polystyrene assay plate (Costar) with the polypeptide overnight at 4°C. in a 200 mM carbonate buffer (Pierce, Rockford Ill.) at a concentration ranging from 0.3 to 30 µg/mL. Unbound polypeptide may be removed by washing with distilled, deionized water and the plates may then be blocked with 1% BSA/carbonate buffer for two hours at room temperature. Plates may then be washed with ~~Tris/Tween~~ Tris/TWEEN® buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% BSA, 1 mM DTT 0.1% ~~Tween20~~ TWEEN® 20 detergent, protease inhibitor cocktail (Boehringer-Mannheim). The other polypeptide may be labeled (e.g., biotinylated) and allowed to bind to the immobilized polypeptide (e.g., solvated in ~~Tris/Tween~~ Tris/TWEEN® buffer and incubated in the assay plates at 4°C. for varying amounts of time). Plates may then be washed

with PBS/0.1% ~~Triton X-100~~ TRITON® X-100 detergent. Binding may be detected by, for example, probing the assay wells with 1 µg/mL Europium-labeled streptavidin (DELFIA; Wallac Oy, Turku, Finland) in DELFIA Assay Buffer/0.1% ~~Triton X-100~~ TRITON® X-100 for one hour at room temperature. Unbound Europium-labeled streptavidin may be removed by washing with PBS/0.1% ~~Triton X-100~~ TRITON® X-100 detergent. Europium may be released for time-resolved fluorescence (TRF) measurements with the DELFIA Enhancement Buffer. TRF measurements may be made, for example, with a DELFIA 1234 (Wallac Oy, Turku, Finland) fluorometer.

Please replace the middle paragraph on page 17 with the following paragraph:

Within similar assays, a radioactive label may be substituted for the biotin. For example, a ³²P-labeled polypeptide may be generated by phosphorylation of a suitable site linked to the WW domain polypeptide or PY motif polypeptide. One such site is the PKA site in the pGEX KG vector (Pharmingen), which may be labeled using α-[³²P]-ATP and protein kinase A (Sigma). The amount of binding may be quantitated by, for example, Cerenkov counting or SDS-PAGE using standard techniques. The solid support used may also be varied. One suitable support for such assays is neutravidin agarose beads (Pierce, Rockford, Ill.). Binding may be performed using such a support by incubation in a PBS/1% ~~Tween20~~ TWEEN® 20 detergent buffer in an end-over shaker at 4°C. for varying amounts of time. It will be apparent that any of these assays may be modified to permit immobilization after binding takes place.

Please replace the middle paragraph on page 18 with the following paragraph:

Other in vitro assays may be designed to assess the effect of an agent on ubiquitination of an E3 ubiquitin ligase and/or a Smad. In vitro ubiquitination reactions are well known in the art. For example, coupled ubiquitination assays (in which ubiquitin transfer from E1 to E2, and from E2 to E3, is monitored) may be employed. Such assays require the reconstitution of an E1/E2/E3 pathway. Recombinant E1 and E2 components are available from a variety of sources (e.g., BostonBiochem, Cambridge, Mass.) for coupling ubiquitin to an E3 ligase of interest. Radiolabeled ubiquitin may be generated using standard techniques, such as PKA-mediated incorporation of [³²P]-phosphate from α-[³²P]-ATP to the PKA site of the GST-ubiquitin fusion protein (pGEX KG expression vector). One suitable ubiquitin assay buffer is: 50 mM Tris pH 7.6, 1 mM ATP, 0.2 mM EDTA, 5 mM MgCl₂, 1 unit inorganic pyrophosphatase, 0.005% ~~Triton X-100~~ TRITON® X-100 detergent and 1 µM staurosporine. In a 0.030 mL reaction, the following amounts of reaction components are generally suitable: 50-200 ng E1, 0.1-1 µg E2, 5 µg GST-ubiquitin (BostonBiochem, Cambridge, Mass.) and 50-200 ng E3. Reactions may be performed at room temperature and terminated with a SDS-PAGE loading buffer that does not contain

mercaptans. Reactions may be analyzed by SDS-PAGE. An assay may be similarly performed with endogenous proteins from, for example, HeLa cell extract fractions (see Hershko et al., J. Biol. Chem. 258:8206-8214, 1983). For measuring Smad protein ubiquitination, a Smad polypeptide is included in the reaction. These assays may be further modified to measure Smad protein degradation by incorporation of 100-1000 ng of 20S proteasome (Boston Biochem, Cambridge, Mass.) into the assay.

Please replace the paragraph bridging pages 29 and 30 with the following paragraph:

Each domain was individually expressed as a GST fusion protein. A TRF binding assay was used to evaluate interactions of PY motif peptides with these domains or GST alone. WW domains were bound to a 96-well polystyrene assay plate (Costar) overnight at 4°C. in a 200 mM carbonate buffer (Pierce, Rockford, Ill.) at different concentrations (0, 1, 3, 10 µg/mL). Unbound WW domain was washed away with distilled, deionized water and the plates were blocked with 1% BSA/carbonate buffer for 2 hours at room temperature. The plates were then washed with ~~Tris/Tween~~ Tris/TWEEN® buffer: 50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% BSA, 1 mM DTT, 0.1% ~~Tween20~~ TWEEN® 20 detergent, protease inhibitor cocktail (Boehringer-Mannheim).

Please replace the third paragraph on page 30 with the following paragraph:

These biotinylated peptides were solvated in ~~Tris/Tween~~ Tris/TWEEN® buffer and added to the assay plates (30 µM). The plates were incubated at 4°C. for varying amounts of capture time. The plates were then washed with PBS/0.1% ~~X100~~ TRITON® X-100 detergent and probed for 1 hour at room temperature with 1 µg/mL Europium-labeled streptavidin (DELFI A; Wallac Oy, Turku, Finland) in DELFI A Assay Buffer/0.1% Triton X100. The unbound Europium-labeled streptavidin was washed with PBS/0.1% ~~X100~~ TRITON® X-100 detergent. Europium was released for time-resolved fluorescence measurements with the DELFI A Enhancement Buffer. Measurements were made on either the DELFI A 1234 or Victor fluorometers.

Please replace the first paragraph on page 32 with the following paragraph:

Recombinant E1 (ubc5c) and E2 (ubc7) components were obtained from BostonBiochem (Cambridge, Mass.). Radiolabeled ubiquitin was generated by PKA-mediated incorporation of [³²P]-phosphate from α-[³²P]-ATP to the PKA site of the GST-Ub fusion protein (pGEX KG expression vector). The ubiquitin assay buffer (UbB) was as follows: 50 mM Tris pH 7.6, 1 mM ATP, 0.2 mM EDTA, 5 mM MgCl₂, 1 unit inorganic pyrophosphatase, 0.005% ~~Triton X100~~ TRITON® X-100 detergent, and 1 µM staurosporine. In a 0.030 mL reaction, the following

components were present: 50-200 ng E1, 0.1-1 μ g E2 and 5 μ g GST-Ub. Reactions were run at room temperature and terminated with a SDS-PAGE loading buffer that did not contain mercaptans. Reactions were analyzed by SDS-PAGE. The ubiquitination of active site cysteine residues of E1 and E2s (ubc Sc and ubc 7) was observed (FIGS. 9A-9C). The addition of 20 mM DTT prevented the formation of the thioester intermediates (FIGS. 9B-9C).